JOURNAL OF BACTERIOLOGY, Apr. 1982, p. 161–167
0021-9193/82/040161-07$02.00/0

Vol. 150, No. 1

Rhizobium japonicum Mutants That Are Hypersensitive to Repression of H_2 Uptake by Oxygen†

ROBERT J. MAIER* AND DAVID M. MERBERG

Department of Biology and McCollum-Pratt Institute, Johns Hopkins University, Baltimore, Maryland 21218

Received 27 July 1981/Accepted 12 November 1981

The synthesis of an H_2 oxidation system in free-living *Rhizobium japonicum* wild-type strain SR is repressed by oxygen. Maximal H_2 uptake rates were obtained in strain SR after derepression in 11 μM or less dissolved oxygen. Oxygen levels above 45 μM completely repressed H_2 uptake in strain SR. Five *R. japonicum* mutant strains that are hypersensitive to repression of H_2 oxidation by oxygen were derived from strain SR. The mutants were obtained by screening H_2 uptake-negative mutants that retained the ability to oxidize H_2 as bacteroids from soybean nodules. As bacteroids, the five mutant strains were capable of H_2 oxidation rates comparable to that of the wild type. The mutants did not take up H_2 when derepressed in 22 μM dissolved oxygen, whereas strain SR had substantial activity at this oxygen concentration. The O_2 repression of H_2 uptake in both the wild-type and two mutant strains, SR174 and SR200, was rapid and was similar to the effect of inhibiting synthesis of H_2 uptake system components with rifampin. None of the mutant strains was able to oxidize H_2 when the artificial electron acceptors methylene blue or phenazine methosulfate were provided. The mutant strains were not sensitive to killing by oxygen, they took up O_2 at rates similar to strain SR, and they did not produce an H_2 uptake system that was oxygen labile. Cyclic AMP levels were comparable in strain SR and the five mutant strains after subjection of the cultures to the derepression conditions.

In the symbiotic association with soybean, *Rhizobium japonicum* possesses an H_2 oxidation system that is responsible for recycling the H_2 produced by nitrogenase. This H_2 oxidation system produces ATP to help support the energy-expensive nitrogenase reaction (10, 23). Alternatively, H_2 oxidation by free-living *R. japonicum* can provide energy to support chemoautotrophic growth of the cells (13, 14). When free-living *R. japonicum* expresses H_2 oxidation activity, concomitant CO_2 fixation occurs via ribulose 1,5-bisphosphate carboxylase (14, 17, 24).

Factors that regulate the synthesis of the H_2 uptake system in free-living *R. japonicum* include H_2, carbon substrates, carbon dioxide, and oxygen (15, 19, 24). The expression of H_2-oxidizing activity in free-living *R. japonicum* is dependent on maintaining low levels of oxygen in the medium (15, 18, 19). Oxygen rapidly represses formation of the H_2 uptake system, and the rapid rate of repression is similar to the effects produced by adding rifampin or chloramphenicol (19). Nothing more is known about regulation of H_2 uptake by oxygen. Chemoautotrophic growth of *R. japonicum* also required that oxygen concentrations be carefully maintained, and optimal growth rates required gradual increases in oxygen partial pressure as the cultures increased in turbidity (14).

The isolation of mutant strains of *R. japonicum* unable to grow chemoautotrophically with H_2 has been previously reported from this laboratory (17). The mutant strains previously characterized were classified into several different groups. These included strains unable to oxidize H_2 (Hup^-) and strains lacking ribulose 1,5-bisphosphate carboxylase activity (17). Further characterization of the Hup^- mutants indicates that some of these strains are able to oxidize H_2 as bacteroids from soybean nodules and in free-living culture require lower oxygen levels than the wild-type parent strain for the expression of the H_2 uptake system.

MATERIALS AND METHODS

Chemicals. Sodium gluconate, L-arabinose, sodium ascorbate, sodium glutamate, rifampin, chloramphenicol, polyvinyl polypyrrolidone, methylene blue, and phenazine methosulfate were obtained from Sigma Chemical Co., St. Louis, Mo. Potassium phosphate for derepression medium and other chemicals for routine culture of *Rhizobium japonicum* were obtained from J. T. Baker Chemical Co., Phillipsburg, N.J.

† Publication no. 1135 from the Department of Biology.

Derepression of the H_2 oxidation system. *R. japonicum* strain SR (20) and mutants derived from strain SR (17) were cultured and derepressed essentially as described previously (17). This derepression involved suspending cells in 0.05 M potassium phosphate buffer (pH 7.0) containing 2.5 mM $MgCl_2$ to a concentration of approximately 4×10^8 cells per ml. Ten milliliters of this suspension was added to a 250-ml prescription bottle, and the bottle was sealed with a serum stopper and flushed with N_2 until no O_2 could be detected amperometrically. Sufficient H_2, CO_2, and O_2 were added to obtain an atmosphere composed of 84% N_2, 10% H_2, 5% CO_2, and 1.0% O_2. The O_2 level was varied from 1.0% in some experiments as described below, and the N_2 partial pressure varied accordingly. A 1.0% O_2 partial pressure corresponded to a dissolved oxygen concentration of 11.3 μM under the conditions we used. The bottles were incubated at 30°C and shaken at 120 cycles per min. During derepression, particularly in the low-O_2 (0.1% O_2 partial pressure) cultures, gas samples were periodically removed from bottles and monitored for oxygen concentration amperometrically, and the O_2 concentration of the bottle was adjusted if necessary.

The derepression experiments were performed by using a small culture volume (10 ml), a large gas phase (240 ml), a relatively low cell concentration (approximately 4×10^8 cells per ml), and shaking cultures so that rapid gas equilibration would occur. In some experiments dissolved O_2 tensions were also monitored amperometrically during derepression and are reported in the text for the various oxygen partial pressures used. Based upon many amperometric measurements of dissolved O_2 levels from cultures rapidly sampled during derepression and comparison with dissolved O_2 standards, we concluded that the dissolved O_2 levels in derepressing cultures were the same as those reported in the text. In our system air-saturated water had an oxygen content of 0.24 mM at 30°C.

H_2 uptake assays. Rates of H_2 uptake were measured amperometrically as described previously (19, 26, 27), with oxygen provided as an electron acceptor. A 5-ml portion of the cell suspension was removed with a syringe from the 250-ml bottle and injected into a serum-stoppered 21-ml vial which was then flushed with N_2. The cells were then transferred by syringe to the 4.5-ml cuvette of the amperometric device, and 62 nmol of O_2 and 37.7 nmol of H_2 (as gas-saturated solutions) were added to the cuvette to initiate the assay. These concentrations were sufficient for saturation of the H_2 uptake reaction. After the H_2 uptake measurement, cell numbers were estimated by comparison of optical density measurements with a standard curve of viable cell number versus optical density at 540 nm.

Growth of soybean plants and harvest of bacteroids. Maryland certified soybean seeds (cultivar Essex) were surface sterilized (25) and germinated in the dark for 48 h. Germinated seedlings were planted 5 seeds per pot into sterile Leonard jar assemblies (25). The Leonard jars contained a mixture of sand and vermiculite and contained N-free sterile nutrient solution (25). Each seed was then inoculated with 0.5 ml of a turbid cell suspension of the appropriate strain, and the seeds were covered with paraffin-coated sand. The Leonard jars were incubated in the greenhouse for 4 weeks with both natural and supplemental light (supplied by 250-W Mercury-Vapor lights; Super Plant Lite; Duro-Lite, Inc.). Supplemental light (85 $\mu E\ M^{-2}\ s^{-1}$, measured at the top of the pot) was supplied only when the natural light intensity dropped below 950 $\mu E\ M^{-2}\ s^{-1}$ as controlled automatically by an electronic photosensor and only for a maximum period of 18 h day^{-1}. Control Leonard jar assemblies containing uninoculated seedlings were checked for nodulation to insure that *R. japonicum* was absent from the seeds or soil mixture. Nodules were picked from the plants and used the same day. Nodules (2 to 4 g from each strain) were crushed in 0.05 M potassium phosphate buffer (pH 6.8) containing 0.2 M sodium ascorbate (5 ml of buffer per g of nodules) and 1 g of acid-washed polyvinylpolypyrrolidone per 3 g of nodules as described previously (10, 11). The bacteroids were then harvested by filtration through cheesecloth and centrifugation (10, 11). Bacteroids were washed twice in 0.05 M potassium phosphate buffer (pH 7.0) containing 2.5 mM $MgCl_2$ (10). Between and after washings the pelleted bacteroids were carefully separated from the polyvinylpolypyrrolidone layer and then suspended in the potassium phosphate buffer. All of these steps were performed under aerobic conditions. H_2 uptake assays were performed amperometrically (26, 27) like those described above for free-living cultures. Bacteroids were suspended in 3% (wt/vol) NaOH and heated at 90°C for 10 min, and protein was determined by the dye-binding method of Bradford (7).

RESULTS

Screening Hup^- mutants for H_2-oxidizing activity as bacteroids from soybean nodules. The isolation of Hup^- *R. japonicum* mutant strains from the Hup^+ parent strain SR was described previously (17). These Hup^- strains were unable to oxidize H_2 in free-living culture when incubated in a carbon-free medium and in an atmosphere containing N_2, H_2, CO_2, and 1.0% partial pressure oxygen. These conditions allowed the parent strain SR to oxidize H_2 at optimal rates.

We wished to determine the H_2-oxidizing abilities of the many free-living Hup^- mutants available in our laboratory as bacteroids isolated from soybean nodules. In two separate experiments each free-living Hup^- mutant was inoculated onto a group of soybean seedlings, and the nodules were harvested. Bacteroids from these nodules were then tested for H_2 oxidation activity. Most of the free-living Hup^- mutants showed little or no activity as bacteroids; however, 13 of 44 (experiment 1) and 9 of 34 (experiment 2) had substantial (1.0 to 3.9 μmol h^{-1} mg of $protein^{-1}$) H_2-oxidizing activity as bacteroids. This result indicated that some factor(s) present in the root nodule allowed expression of H_2 oxidation in strains apparently lacking this capability in free-living culture.

Repression of H_2 oxidation by oxygen. Previous studies showed that a high level of oxygen

(partial pressure, 20%) repressed the formation of the H_2 oxidation system in nonshaking cultures of *R. japonicum* strain SR (19). The effect of raising the O_2 partial pressure above shaking derepressing cultures from the optimal level of 1.0% O_2 (dissolved O_2 concentration, 11 μM) to 4.0% O_2 (dissolved oxygen concentration, 45 μM) 8 h after subjection of the culture to derepressing conditions is shown in Fig. 1. When the oxygen concentration was increased to 45 μM (arrow), further derepression ceased abruptly. The addition of rifampin or chloramphenicol (chloramphenicol data not shown) to suspensions after the derepression was initiated (Fig. 1) resulted in inhibition of H_2 oxidation system synthesis in a pattern similar to that observed by O_2 repression. Sparging of previously derepressed cultures of SR with 100% oxygen did not inhibit H_2 oxidation activity.

Repression of H_2 oxidation by oxygen in mutant strains. Since the nodule contains the oxygen-binding protein leghemoglobin, the free dissolved oxygen concentration at the surface of *R. japonicum* bacteroids has been estimated to be approximately 0.01 μM (5, 28). We reasoned

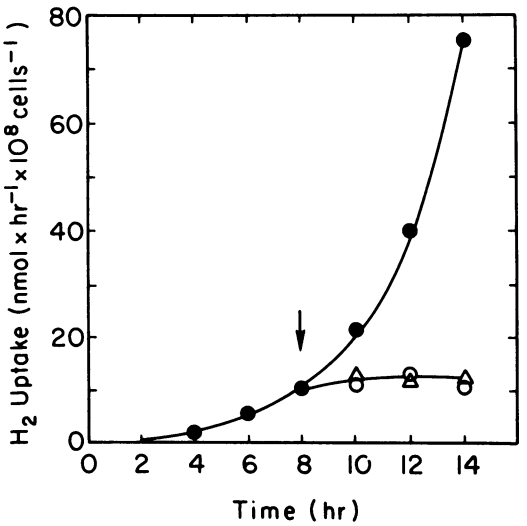

FIG. 1. Effect of raising the O_2 partial pressure on derepression of H_2 oxidation in wild-type strain SR. Cultures were derepressed as described in the text in an atmosphere composed of 84% N_2, 10% H_2, 5% CO_2, and 1% O_2. At 8 h (arrow), additional O_2 was injected to bring the atmosphere to 4% partial pressure oxygen (○), rifampin (25 μg ml^{-1}) was added (△), or no addition was made (●). Activity was monitored at 10, 12, and 14 h. Each point is the average of the H_2 uptake activities from two individual derepressed cultures. H_2 uptake was monitored amperometrically with oxygen provided as electron acceptor as described in the text. The addition of chloramphenicol (25 μg ml^{-1}) at 8 h gave results comparable to that of rifampin.

TABLE 1. Hydrogen uptake after derepression in two different oxygen partial pressures and oxygen uptake activities of *R. japonicum* strains[a]

Strain	H_2 uptake activity (nmol of gas h^{-1} 10^8 $cells^{-1}$) after derepression in:		O_2 uptake activity (nmol of gas h^{-1} 10^8 $cells^{-1}$) after derepression in 2.0% O_2
	0.4% O_2	2.0% O_2	
SR	72	67	31
SR200	42	<1.0	28
SR178	9	<1.0	38
SR186	15	1.8	29
SR119	11	<1.0	36
SR174	12	<1.0	33
SR180	<1.0	<1.0	NT[b]
SR129	<1.0	<1.0	NT

[a] Strains were derepressed for 24 h in an atmosphere composed of 10% H_2, 5% CO_2, and the indicated oxygen concentration; the balance of the atmosphere was N_2. After derepression cells were assayed for H_2 uptake amperometrically with oxygen as the terminal electron acceptor as described in the text. O_2 uptake activity was measured amperometrically in the absence of H_2 in the cultures derepressed in 2.0% partial pressure O_2. O_2 (62 nmol) was injected into the cuvette of the amperometric device to initiate the assay. Each value in the table is the average of the activities from two individual derepressed cultures.

[b] NT, Not tested.

that some of the mutant strains which expressed H_2 oxidation activity as bacteroids, but not as free-living cells, might be hypersensitive to repression by O_2. Therefore the mutant strains that contained substantial levels of activity as bacteroids were screened for H_2 oxidation activity after derepression in 2.0% partial pressure (23 μM) versus 0.4% partial pressure (4.5 μM) dissolved oxygen (Table 1). The parent strain SR contained high levels of H_2 oxidation activity at both O_2 levels. Some of the mutant strains that did not contain H_2 oxidation activity at the high oxygen level were able to oxidize H_2 when derepressed at a lower (4.5 μM) dissolved oxygen level. Control experiments ruled out the possibility that the addition of O_2 and H_2 utilizing suspensions of these mutants inactivated the H_2 uptake system. Some other mutant strains that contained substantial activity as bacteroids (see below) did not appear to be hypersensitive to oxygen (for example, SR180 and SR129 in Table 1). We have not observed H_2 oxidation in these strains even at dissolved oxygen concentrations as low as 0.5 μM.

It was possible that the mutants that were hypersensitive to oxygen repression of H_2 uptake had higher respiratory rates than the parent strain; therefore they would take up more oxygen than the parent strain. However, oxygen uptake rates were similar for the parent strain

SR and for the mutants (Table 1). In addition, the possibility of a general O_2 killing effect was ruled out by determining viable cell numbers after derepression of the cells for 20 h in 23 μM dissolved oxygen (the high-O_2 condition used for the results reported in Table 1). The viability (based on plate counts) of the mutants listed in Table 1 and strain SR were approximately the same after the 20-h incubation period. Also, all of the mutants grew as well as the parent type SR in routine liquid culture medium (6) in air. Other evidence we can cite to indicate that the O_2 repression effect is not merely a general poisoning of cellular metabolism was the similar respiratory rates (O_2 uptake) of the mutants and the wild type (Table 1). Experiments involving sparging of H_2 oxidizing cultures of the mutants with O_2 ruled out the possibility that the mutants produced an O_2-labile H_2 oxidation system.

H_2 oxidation in bacteroids of the mutants. The mutants that were tested for hypersensitivity to oxygen repression were categorized as containing substantial H_2 uptake activity as bacteroids in the initial screening experiment. We repeated measurements of the symbiotic H_2 oxidation capabilities of these strains (Table 2). All of the mutant strains listed in Table 1 that were hypersensitive to oxygen repression in free-living culture, strains SR200, SR178, SR119, SR174, and SR186, as bacteroids retained H_2 oxidizing ability comparable to that of the parent strain SR. This result is not surprising due to the low levels of oxygen assumed to be present at the bacteroid surface.

TABLE 2. H_2 uptake activity in bacteroids of *R. japonicum* strain SR and mutant strains[a]

Strain	H_2 uptake activity (μmole h^{-1} mg of protein^{-1})
SR	3.5
SR200	3.1
SR178	3.8
SR119	2.7
SR174	2.8
SR186	3.0
SR180	3.1
SR129	2.7
SR182	<0.1
SR194	<0.1

[a] Details of the growth of the soybeans, harvest of the bacteroids from nodules, and assay of the bacteroids are described in the text. All mutant strains were Hup$^-$ in free-living culture when derepressed for 24 h in an atmosphere composed of 83% N_2, 5% CO_2, 10% H_2, and 2% O_2. The minimum detectable level of H_2 oxidation was judged to be 0.1 μmol h^{-1} per mg of protein. H_2 uptake was determined amperometrically with O_2 provided (62 nmol) as described in the text for assay of free-living cells.

The bacteroid H_2 uptake activities of two other mutant strains, SR180 and SR129, are shown in Table 2. These strains do not have H_2 uptake activity in free-living culture even when they are subjected to derepression under low O_2 conditions (Table 1). However, they contain H_2 uptake activity comparable to that of the wild type as bacteroids. Examples of other mutant strains are represented by strains SR182 and SR194 in Table 2. These mutant strains exemplify the phenotype of most Hup$^-$ mutants; they are Hup$^-$ both in free-living culture and as bacteroids from nodules.

Further examination of mutants SR200 and SR174. We wanted to compare the kinetics of repression of H_2 oxidation by O_2 in the mutants with that in the parent strain SR. Cultures of SR200 and SR were derepressed in 1.1 μM dissolved oxygen and after 8 h were challenged with a higher level of O_2. Levels of H_2 oxidation were then monitored with time as in the experiment described for the wild-type SR in Fig. 1. The results are shown in Fig. 2. A 1.1 μM level of dissolved oxygen allowed derepression to proceed in both strain SR200 and the wild type, SR. However, after raising the oxygen concentration to 23 μM at 8 h after the start of derepression (arrow in Fig. 2), strain SR200 failed to continue derepresison, whereas strain SR continued to increase in H_2 uptake specific activity.

In strain SR, the repression of H_2 oxidation by O_2 is rapid and parallels the inhibition of enzyme formation by the addition of rifampin (Fig. 1). To determine whether repression by O_2 in the mutants was similar to that in the parent strain SR, an experiment analogous to that performed on strain SR in Fig. 1 was carried out. The derepression kinetics of H_2 oxidation was followed in two mutant strains, SR174 and SR200, and the inhibitory effects of raising the oxygen concentration to 23 μM was compared to that of adding rifampin (Fig. 3). Both mutant strains SR200 (Fig. 3A) and SR174 (Fig. 3B) derepressed normally in 1.1 μM dissolved oxygen. When the oxygen level available to the cultures of either mutant was increased to 23 μM further derepression ceased. The kinetics of repression for both mutants was similar to that observed by inhibiting mRNA synthesis by adding rifampin at 8 h. This is similar to the result that was observed for the parent strain SR, with the exception that higher O_2 levels were needed for repression of H_2 oxidation in strain SR (Fig. 1).

Cyclic AMP levels in strain SR and the mutants. Cyclic AMP has been postulated to be one factor that can regulate H_2 oxidation in free-living *R. japonicum* (16). Therefore, it was possible that the mutant strains underproduced cyclic AMP. Cyclic AMP levels and H_2 oxidation rates were measured in strain SR and in the

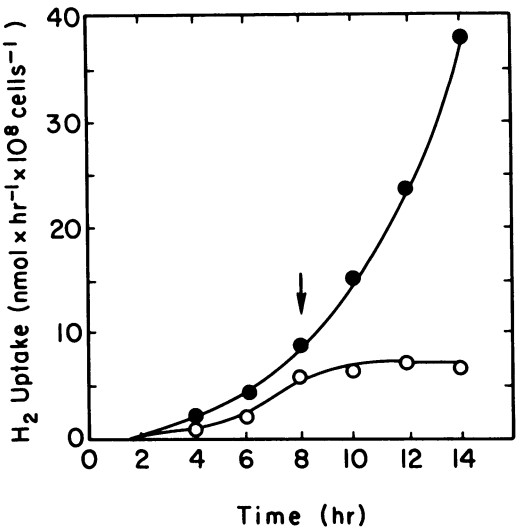

FIG. 2. Effect of raising the O_2 partial pressure on derepression of H_2 oxidation in wild-type strain SR and mutant strain SR200. Cultures were derepressed as described in the text in an atmosphere composed of 84.9% N_2, 10% H_2, 5% CO_2, and 0.1% O_2. Gas samples were periodically removed, the O_2 concentration was monitored by use of an oxygen electrode, and the O_2 level was readjusted to 0.1% if necessary. This corresponded to a dissolved oxygen concentration of approximately 1.0 μM. At 8 h (arrow) the O_2 partial pressure was raised to 2.0% (dissolved oxygen of 23 μM) in cultures of both strains, and subsequent H_2 uptake rates were monitored at 10, 12, and 14 h. Each point is the average of the H_2 uptake activities from two individual derepressed cultures: SR (●) and SR200 (○).

mutant strains after subjection of the cells to derepressing conditions in 23 μM dissolved O_2 for 16 h (Table 3). Intracellular cyclic AMP levels were not lower in any of the mutants than in the parent strain SR. Extracellular cyclic AMP levels were also determined and found to be no lower in any of the mutants than in the wild type (data not shown).

Assay for H_2 oxidation with artificial electron acceptors. The complete H_2 oxidation system is composed of an H_2-activating hydrogenase and other unidentified electron transport components. The purified H_2-activating hydrogenase from *R. japonicum* bacteroids can couple H_2 uptake to the reduction of artificial electron carriers, such as methylene blue and phenazine methosulfate (3). Free-living *R. japonicum* strain SR will oxidize H_2 in the absence of oxygen if these artificial electron acceptors are provided (17). We wanted to determine whether the mutants, after subjection to the conditions of oxygen repression of H_2 oxidation, were able to oxidize H_2 with these artificial electron acceptors. Strain SR and mutant strains SR174, SR200, SR186, and SR119 were subjected to the derepressing conditions in 2.0% partial pressure oxygen for 24 h. H_2 uptake rates were then measured with oxygen, methylene blue, or phenazine methosulfate as the electron acceptor. Whereas the parent strain SR was able to oxidize H_2 with all three electron acceptors, the mutants were not able to oxidize H_2 with any of the acceptors provided. This result indicates that the mutants lack the H_2-activating hydrogenase.

DISCUSSION

Mutant strains of *R. japonicum* have been isolated which do not express H_2 oxidation activity at an O_2 level which allows derepression in the wild type. However these strains do produce the H_2 uptake system under lower oxygen levels. The O_2 repression in both the wild type and the mutant strains is rapid and is similar to the effect of inhibiting H_2 oxidation synthesis by adding rifampin or chloramphenicol. The mutant strains are capable of H_2 oxidation rates comparable to that of the wild type when they are isolated as bacteroids from soybean nodules. H_2 uptake expression is probably allowed symbiotically due to the low O_2 environment in the nodule that is provided by the O_2-binding protein leghemoglobin.

By comparison of the mutants with the wild type we conclude that the observed phenotype of the five mutant strains cannot be explained by any of the following: a deficiency in cyclic AMP production, a more active O_2 uptake system, the synthesis of an O_2-labile H_2 uptake system, or a

TABLE 3. Cyclic AMP levels in strain SR and mutant strains of *R. japonicum*[a]

Strain	H_2 uptake activity (nmol h^{-1} 10^8 $cells^{-1}$)	Intracellular cyclic AMP (pmol 10^8 $cells^{-1}$)[b]
SR	32.6	0.06 ± 0.01
SR119	<1.0	0.10 ± 0.04
SR174	<1.0	0.05 ± 0.01
SR178	<1.0	0.08 ± 0.01
SR186	<1.0	0.06 ± 0.02
SR200	1.1	0.09 ± 0.04

[a] Strains were derepressed for 16 h under an atmosphere composed of 10% H_2, 5% CO_2, 2% O_2, and 83% N_2 as described in the text. Five 0.5-ml samples of each strain were withdrawn anaerobically and quickly filtered through 0.45-μm nitrocellulose filters. Cyclic AMP was then determined by radioimmunoassay as described by Lim and Shanmugam (16) with a cyclic AMP assay kit (New England Nuclear Corp., Boston, Mass.). Cells were assayed for H_2 uptake amperometrically with O_2 as the terminal electron acceptor as described in the text.

[b] Mean ± standard deviation for five replicate samples.

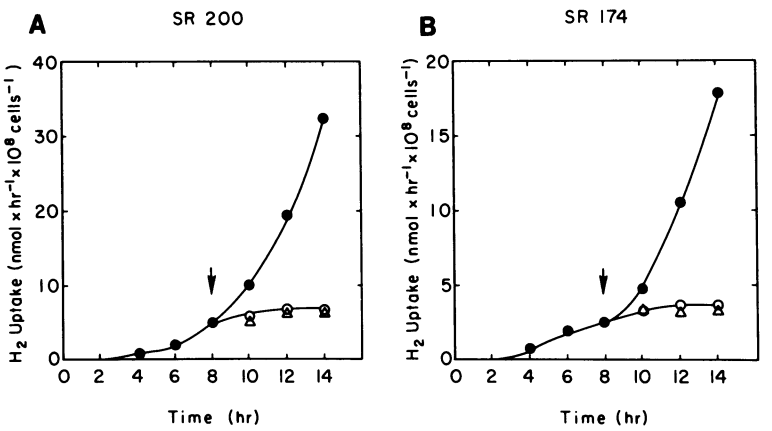

FIG. 3. Effect of raising the O_2 partial pressure on derepression of H_2 oxidation in mutant strains SR200 and SR174. All conditions were like that described in the legend to Fig. 2. Cells were derepressed in 0.1% partial pressure O_2 for 8 h, and the O_2 partial pressure was raised to 2.0% at 8 h (○), rifampin (25 μg/ml) was added at 8 h (△), or no addition was made at 8 h (●). Each point is the average of the H_2 uptake activities from two individual derepressed cultures.

general sensitivity of the strains to O_2 killing. The mutant strains appear to be more sensitive to repression of the H_2 oxidation system by O_2. Regulation of enzyme synthesis by O_2 has been observed for a number of bacterial enzyme systems, including electron transport pathway components (12). Nitrogenase synthesis is also regulated by oxygen in some N_2-fixing bacteria (8, 22). In the H_2-oxidizing bacterium *Aquaspirillum autotrophicum* high levels of oxygen also repressed synthesis of the H_2 oxidation system by an unknown mechanism (2). Although H_2 uptake-negative strains of H_2-oxidizing bacteria have been described, to our knowledge none that are hypersensitive to repression by oxygen have been reported.

Some of the mutant strains previously classified as Hup^- in free-living culture have now been shown to have the capability of expressing H_2 uptake when derepressed in low oxygen concentrations. Among the many remaining mutant strains that are Hup^- in free-living cultures most also lack H_2 uptake activity as bacteroids from nodules. This suggests that the genetic loci for free-living H_2 uptake are the same required for H_2 uptake in the root nodule. The knowledge that some symbiotically Hup^+ strains can express H_2 uptake in free-living culture only when derepressed in low oxygen may be important when screening naturally occurring strains for this capacity for agricultural use.

Oxygen apparently plays a key role in the expression of symbiotic properties in *Rhizobium* spp. Symbiotic characteristics that are expressed in free-living culture only when the cells are subjected to low levels of O_2 include nitrogenase, H_2 oxidation, high levels of heme-synthesizing enzymes (4), bacteroid-like cytochromes (1, 4, 9), and differentiation into bacteroid-like morphology (21). We do not yet know if the mutant strains described here are also hypersensitive to O_2 repression of these other symbiotic characteristics. There are several possibilities for the defect in these mutants. These include the underproduction of a positive regulatory factor made in response to low O_2 and the overproduction of an O_2-sensing factor that negatively regulates the synthesis of H_2 oxidation system components. Further biochemical characterization of the mutants should provide information on the mechanism of regulation by oxygen in *Rhizobium japonicum*.

ACKNOWLEDGMENTS

This work was supported by grant 59-2243-0-1-435-0 from the United States Department of Agriculture Science and Education Administration Competitive Grants Program.

We thank Fred Moshiri, Tom Fynan, Sidney Yoon, and Wes Stites for assistance in various portions of this work.

LITERATURE CITED

1. **Appleby, C. A.** 1969. Electron transport systems of *Rhizobium japonicum*. II. *Rhizobium* hemoglobin;, cytochromes, and oxidases in free-living (cultured) cells. Biochim. Biophys. Acta **172**:88–105.
2. **Aragno, M., and H. G. Schlegel.** 1978. Physiological characterization of the hydrogen bacterium *Aquaspirillum autotrophicum*. Arch. Microbiol. **116**:221–229.
3. **Arp, D. J., and R. H. Burris.** 1979. Purification and properties of the particulate hydrogenase from the bacteroids of soybean root nodules. Biochim. Biophys. Acta **570**:221–230.
4. **Avissar, Y. J., and K. D. Nadler.** 1979. Stimulation of tetrapyrrole formation in *Rhizobium japonicum* by restricted aeration. J. Bacteriol. **135**:782–789.
5. **Bergersen, F. J.** 1980. Leghemoglobin, oxygen supply, and nitrogen fixation: studies with soybean nodules, p. 139–160. *In* W. D. P. Stewart and J. R. Gallon (ed.),

Nitrogen fixation. Academic Press, Inc., New York.
6. **Bishop, P. E., J. G. Guevarra, J. A. Engelke, and H. J. Evans.** 1976. Relation between gluatimine synthetase and nitrogenase activities in the symbiotic association between *R. japonicum* and *Glycine max*. Plant Physiol. **57**:542–546.
7. **Bradford, M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye bindings. Anal. Biochem. **72**:248–254.
8. **Brill, W. J.** 1980. Biochemical genetics of nitrogen fixation. Microbiol. Rev. **44**:449–467.
9. **Daniel, R. M., and C. A. Appleby.** 1972. Anaerobic-nitrate, symbiotic, and aerobic growth of *Rhizobicum japonicum*: effects on cytochrome P-450 and other haemoproteins, nitrate and nitrite reductase. Biochim. Biophys. Acta **275**:347–354.
10. **Emerich, D. W., T. Ruiz-Argueso, T. M. Ching, and H. J. Evans.** 1979. Hydrogen dependent nitrogenase activity and ATP formation in *Rhizobium japonicum* bacteroids. J. Bacteriol. **137**:153–160.
11. **Evans, H. J., B. Koch, and R. Klucas.** 1972. Preparation of nitrogenase from nodules and separation into components. Methods Enzymol. **24**:470–476.
12. **Haddock, B. A., and C. W. Jones.** 1977. Bacterial respiration. Bacteriol. Rev. **41**:47–99.
13. **Hanus, F. J., R. J. Maier, and H. J. Evans.** 1979. Autotrophic growth of H_2 uptake positive strains of *Rhizobium japonicum* in an atmosphere supplied with hydrogen gas. Proc. Natl. Acad. Sci. U.S.A. **76**:1788–1792.
14. **Lepo, J. E., F. J. Hanus, and H. J. Evans.** 1979. Chemoautotrophic growth of hydrogen-uptake positive strain of *Rhizobium japonicum*. J. Bacteriol. **141**:664–670.
15. **Lim, S. T.** 1979. Determination of hydrogenase in free-living cultures of *Rhizobium japonicum* and energy efficiency of soybean nodules. Plant Physiol. **62**:609–611.
16. **Lim, S. T., and K. T. Shanmugam.** 1979. Regulation of hydrogen utilization in *Rhizobium japonicum* by cyclic AMP. Biochim. Biophys. Acta **584**:479–492.
17. **Maier, R. J.** 1981. *Rhizobium japonicum* mutant strains unable to grow chemoautotrophically with H_2. J. Bacteriol. **145**:533–540.
18. **Maier, R. J., N. E. R. Campbell, F. J. Hanus, F. B. Simpson, S. A. Russell, and H. J. Evans.** 1978. Expression of hydrogenase in free-living *Rhizobium japonicum*. Proc. Natl. Acad. Sci. U.S.A. **75**:3358–3262.
19. **Maier, R. J., F. J. Hanus, and H. J. Evans.** 1979. Regulation of hydrogenase in *Rhizobium japonicum*. J. Bacteriol. **137**:824–829.
20. **Maier, R. J., J. R. Postgate, and H. J. Evans.** 1978. *Rhizobium japonicum* mutants unable to use H_2. Nature (London) **276**:494–496.
21. **Pankhurst, C. E., and A. S. Craig.** 1978. Effect of oxygen concentration, temperature and combined nitrogen on the morphology and nitrogenase activity of *Rhizobium* sp. strains 32H1 in agar culture. J. Gen. Microbiol. **106**:207–219.
22. **Robson, R., and J. R. Postgate.** 1980. Oxygen and hydrogen in biological nitrogen fixation. Annu. Rev. Microbiol. **34**:183–208.
23. **Ruiz-Argueso, T., D. W. Emerich, and H. J. Evans.** 1979. Hydrogenase system in legume nodules: a mechanism for providing nitrogenase with energy and protection from oxygen damage. Biochem. Biophys. Res. Commun. **86**:259–264.
24. **Simpson, F. B., R. J. Maier, and H. J. Evans.** 1979. Hydrogen-stimulated CO_2 fixation and coordinate induction of hydrogenase and ribulose bisphosphate carboxylase in a H_2 uptake positive strain of *Rhizobium japonicum*. Arch. Microbiol. **123**:1–8.
25. **Vincent, J. M.** 1970. A manual for the practical study of root-nodule bacteria. International Biological Programme, Blackwell Scientific Publications, Oxford, England.
26. **Wang, R. T.** 1980. Amperometric hydrogen electrode. Methods in Enzymol. **69**:409–412.
27. **Wang, R., F. P. Healy, and J. Meyers.** 1971. Amperometric measurement of hydrogen evolution in chlamydomonas. Plant Physiol. **48**:108–110.
28. **Wittenberg, J. B., F. J. Bergersen, C. A. Appleby, and G. L. Turner.** 1974. Facilitated oxygen diffusion: the role of leghemoglobin in nitrogen fixation by bacteroids isolated from soybean root nodules. J. Biol. Chem. **249**:4057–4066.